A Fluorescence High Throughput Screening Method for the Detection of Reactive Electrophiles as Potential Skin Sensitizers

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Abbreviations*

* *ACN*, Acetonitrile; *AOP*, Adverse Outcome Pathway; *BQ*, Benzoquinone; *CDNB*, 1-chloro-2,4-dinitrobenzene; *COUM*, Coumarin; *Cys-DPRA*, Cysteine-Direct Peptide Reactivity Assay; *DBN*, 1,5-diazabicyclo[4.3.0]non-5-ene; *DCYA*, Dansyl cysteamine; *(DCYA)₂*, Dansyl cysteamine dimer; *DMSO*, dimethyl sulfoxide; *DPRA*, Direct Peptide Reactivity Assay; *ESI*, Electron Spray Ionization; *HPLC*, High Performance Liquid Chromatography; *HR-MS*, High Resolution Mass Spectroscopy; *HTS*, High Throughput Screening; *LLNA*, Local Lymph Node Assay; *Lys-DPRA*, Lysine-Direct Peptide Reactivity Assay; *ML*, Massoia Lactone; *NMR*, Nuclear Magnetic Resonance; *OECD*, Organisation for Economic Co-operation and Development; *P*, Parthenolide; *pd*, peptide depletion; *R_f*, Retention factor; *RFU*, Relative Fluorescence Unit; *RI*, Reactivity Index; *TLC*, Thin Layer Chromatography.

Abstract

Skin sensitization is an important toxicological end-point in the risk assessment of chemical allergens. Because of the complexity of the biological mechanisms associated with skin sensitization, integrated approaches combining different chemical, biological and *in silico* methods are recommended to replace conventional animal tests. Chemical methods are intended to characterize the potential of a sensitizer to induce earlier molecular initiating events. The presence of an electrophilic mechanistic domain is considered one of the essential chemical features to covalently bind to the biological target and induce further haptenation processes. Current *in chemico* assays rely on the quantification of unreacted model nucleophiles after incubation with the candidate sensitizer. In the current study, a new fluorescence-based method, 'HTS-DCYA assay', is proposed. The assay aims at the identification of reactive electrophiles based on their chemical reactivity toward a model fluorescent thiol. The reaction workflow enabled the development of a High Throughput Screening (HTS) method to directly quantify the reaction adducts. The reaction conditions have been optimized to minimize solubility issues, oxidative side reactions and increase the throughput of the assay while minimizing the reaction time, which are common issues with existing methods. Thirty-six chemicals previously classified with LLNA, DPRA or KeratinoSensTM were tested as a proof of concept. Preliminary results gave an estimated 82% accuracy, 78% sensitivity, 90% specificity, comparable to other *in chemico* methods such as Cys-DPRA. In addition to validated chemicals, six natural products were analyzed and a prediction of their sensitization potential is presented for the first time.

Key words: Skin sensitization, Electrophiles, *In chemico* alternative methods, Fluorescence assay, High Throughput Screening

Introduction

The assessment of skin sensitization potential is an important toxicological end-point in the safety evaluation of chemicals intended for use as topical formulations of drugs, cosmetics, fragrances and other personal-care products. Skin sensitization is a complex form of immunotoxicity involving numerous molecular pathways which are highly controlled at the cellular level. For this reason, the development of *ex vivo* methods suitable as stand-alone alternatives to animal tests is challenging. The list of validated animal methods includes the Guinea Pig Maximization Test (GPMT) (Magnusson and Kligman, 1969), the Buehler's test (BT) (Buehler, 1965) and the Local Lymph Node Assay (LLNA) (Kimber and Basketter, 1992; ICCVAM, 2009). The need for new, non-animal alternatives has been reinforced by the introduction in the European Union (EU), and soon worldwide, of new regulations (such as the Registration, Evaluation, Authorization and Restriction of Chemicals, REACH, enacted in 2007) requiring a minimal use of animals for toxicological screenings. In 2012, a new program on the development of Adverse Outcome Pathways (AOP) was launched by the Organisation for Economic Co-operation and Development (OECD). The AOP approach relies on the use of mode of action to understand and predict the potential toxicological effects of chemicals and on the integration of data from chemical, *in vitro* and computational models for risk assessment.

Among the *in vitro* alternatives proposed for skin sensitization (Azam *et al.*, 2006; Python *et al.*, 2007; Natsch and Emter, 2008; Ade *et al.*, 2009; Natsch, 2010; Vandebriel *et al.*, 2010; Nukada *et al.*, 2012; Ramirez *et al.*, 2014, Chittiboyina *et al.*, 2015), the antioxidant response element (ARE)-Nrf2 Luciferase Test Method (KeratinoSensTM) was the first non-animal assay to be adopted by international guideline TG 442D (OECD, 2015b). Other methods under evaluation include the LuSens assay (based on the activity evaluation of a luciferase gene under the control of an ARE promoter), the Myeloid U937 Skin Sensitization Test (MUSST, which measures the upregulation of CD86 in human cell line) (Ade *et al.*, 2006) and the human Cell Line Activation Test (hCLAT, which measures upregulation of cell surface antigens in human monocytic leukemia cell line as a surrogate for dermal dendritic cells) (Ashikaga *et al.*, 2006; Natsch *et al.*, 2013; Natsch *et al.*, 2014). The Direct Peptide Reactivity Assay (DPRA) (Gerberick

et al., 2004a; Gerberick *et al.*, 2007) has been the first *in chemico* test to complete the validation process by international agencies (OECD, 2015a) (Urbisch *et al.*, 2015), although similar tests are expected to advance within the evaluation process in the near future. The rationale of the chemical methods proposed so far is based on the hypothesis that skin sensitizers covalently bind to a nucleophilic biological target in order to elicit an allergic response. Known sensitizers have been classified into five mechanistic domains: Michael acceptors, acylating agents, Schiff base initiators, pre- or pro-electrophiles (non-electrophiles) and electrophiles capable of nucleophilic substitution (S_N1 , S_N2 and S_NAr) (Aptula *et al.*, 2005). Several model nucleophiles (Schwöbel *et al.*, 2011) have been proposed for categorization purposes, mainly based on cysteine or lysine as reactive amino acids. The identification and classification of chemical sensitizers using DPRA are based on HPLC quantification of the reaction between nucleophilic peptides and potential sensitizers. The potency of the sensitizer is determined based on the percentage of peptide depletion (*pd*) assuming that peptide depletion is directly proportional to the potency of the test chemical. The DPRA method has been further refined to differentiate between adduct formations and auto-dimerization induced by some skin sensitizers (Natsch and Gfeller, 2008). A High-Throughput Kinetic Profiling (HTKP) method has been subsequently proposed (Roberts and Natsch, 2009) to address some of the drawbacks associated with peptide reactivity assays. Naphthalene derivatives of specific amino acids have been introduced in the Amino acid Derivative Reactivity Assay (ADRA) to increase the precision of HPLC quantifications (Fujita *et al.*, 2014; Yamamoto *et al.*, 2015).

The peptide-based assays rely on the estimation of reactivity through the quantification of the unreacted peptide and may suffer from solubility issues associated with the different chemical nature of the hydrophilic nucleophile and hydrophobic sensitizers. Because of the poor water solubility of the majority of skin allergens, drowning-out effects (a precipitation phenomenon which may occur after mixing a solution with another solvent not compatible with the solute) have been reported which may affect the quantification of the reaction and the resulting classification (Natsch and Gfeller, 2008).

In the present study, a novel *in chemico* High Throughput Screening (HTS) to rapidly identify potential electrophilic skin sensitizers using a fluorescent cysteamine derivative is proposed. The

described Dansyl Cysteamine Assay (DCYA) assay relies on the direct quantification of the formation of hapten-thiol adducts by fluorescence detection in multi-well microplates (PCT 106 application filed, PCT/US15/38142). The proposed method has been tested on 36 sensitizers selected from a compilation of validated data (Natsch *et al.*, 2013). The compounds were chosen based on their chemical diversity across different sensitization potency (non-sensitizers, weak, moderate and strong, including pre- and pro-haptens) as a proof of concept. To assess the scope of the developed method, three known human allergens plus three potential sensitizers (for which no data from either LLNA or alternative methods are yet available) were also tested and *in chemico* categorization results are here reported for the first time.

Materials and Methods

Caution: All tested compounds may cause skin allergy. These compounds must be handled with care.

Chemicals and reagents

Cystamine dihydrochloride 96% (CAS # 56-17-7), dansyl chloride $\geq$ 99% (CAS #605-65-2), diphenyl cyclopropanone, *p*-benzoquinone, 1-chloro-2,4-dinitrobenzene, *p*-hydroquinone, propionolactone, 3-hydroxytyrosol, 1,2-cyclohexanedicarboxylic anhydride, 2-methyl-4-isothiazolin-3-one, cinnamaldehyde, 2,4-heptadienal, 4-hex-3-en-one, squaric acid, *trans*-2-hexenal, resorcinol, diethyl maleate, lilial, cinnamyl alcohol, *cis*-6-nonenal, 5-methyl-2,3-hexanedione, ethyl acrylate, aniline, 1-bromobutane, vanillin, tartaric acid, chlorobenzene, lactic acid, salicylic acid, coumarin, benzaldehyde, citral, farnesal, safranal costunolide, alantolactone, parthenolide, oxalic acid, benzyl benzoate, standardized buffer solution pH 7 and 1,5-diazabicyclo[4.3.0]non-5-ene 98% (DBN) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Massoia lactone, *trans*-2-pentenal, L-(-)-carvone, (-)-perillaldehyde and nootkatone were kindly donated by Citrus and Allied Essences Ltd. (Lake Success, NY, USA). The CAS registry numbers of tested sensitizers are given in Table 1. Polymer-supported maleimide (SiliaBond® Maleimide, $\geq$ 0.64 mmol/g) was purchased from SiliCycle (Quebec City, Quebec, Canada). Standardized buffer solution pH 10 ± 0.02 (cat. # SB116-500), microcentrifuge tubes,

polypropylene solvent-resistant 96-well microplates and TLC plates (silica gel supported on aluminum sheets, without fluorescence indicator) were purchased from Fisher Scientific (Suwanee, GA, USA).

N,N'-(Disulfanediy)bis(ethane-2,1-diyl))bis(5-(dimethylamino)naphthalene-1-sulfonamide)
(DCYA disulfide)

In a 500 mL round bottom flask, dansyl chloride (4.03 g, 14.94 mmol) was dissolved in a solution of 170 mL of acetone:water (30:1). A solution of cystamine dihydrochloride (1.61 g, 7.15 mmol, 0.49 equiv.) in 80 mL 0.1 M aqueous NaHCO₃ was added drop wise. The solution was maintained at pH 7.5 by addition of aqueous 0.5 M sodium hydroxide. After 90 min, the reaction mixture was diluted with 100 mL of chloroform, and the resulting solution was washed four times with aqueous sodium bicarbonate, followed by water. The organic layer was dried over anhydrous MgSO₄, concentrated and purified by column chromatography (silica gel, gradient elution acetone 10% to 40% in hexane) to yield 4.14 g of DCYA disulfide as a fluffy, yellow solid (6.70 mmol, 89.6%), TLC R_f = 0.25, 30% acetone in hexanes, mp 71-72 °C. IR ν_{max} (cm⁻¹) 3280, 2942, 1574, 1439, 1408, 1311, 1140, 1063, 789. ¹H-NMR: (500 MHz, CDCl₃) δ 8.54 (d, *J* = 8.5 Hz, 1H), 8.27 – 8.20 (m, 2H), 7.53 (dt, *J* = 8.6, 7.4 Hz, 2H), 7.17 (d, *J* = 7.6 Hz, 1H), 5.24 (t, *J* = 6.2 Hz, 1H), 3.09 (q, *J* = 6.3 Hz, 2H), 2.88 (s, 6H), 2.48 (t, *J* = 6.3 Hz, 2H). ¹³C-NMR: (126 MHz, CDCl₃) δ 152.2, 134.5, 130.8, 130.0, 129.8, 129.6, 128.7, 123.3, 118.7, 115.4, 45.6, 41.7, 37.9. ESI (-) MS *m/z* 617.2 [M - H]⁻.

5-(Dimethylamino)-N-(2-mercaptoethyl)naphthalene-1-sulfonamide (DCYA)

In a 50 mL round bottom flask, the DCYA disulfide compound (0.32 g, 0.524 mmol) was dissolved in tetrahydrofuran (18 mL) and water (2 mL), cooled to 0 °C and sodium borohydride (0.20 g, 5.29 mmol) was added in small portions. After 4 h, the solvent was evaporated and the residue was diluted with water and extracted with diethyl ether (3x 10 mL). The combined organic layers were dried over anhydrous MgSO₄, concentrated and purified on silica gel (gradient elution 10% to 30% acetone in hexane) to yield DCYA thiol as greenish-yellow fluffy solid (0.27 g, 0.87 mmol, 83%, TLC R_f = 0.40 on

silica gel plates, 30% ethyl acetate in hexane). IR ν_{max} (cm⁻¹) 3256, 2949, 1301, 1136, 1090, 940, 845, 786. ¹H NMR (500 MHz, CDCl₃) δ 8.56 (d, J = 8.5 Hz, 1H), 8.28 (d, J = 8.6 Hz, 1H), 8.25 (dd, J = 7.3, 1.1 Hz, 1H), 7.58 (t, J = 7.6 Hz, 1H), 7.53 (dd, J = 8.4, 7.4 Hz, 1H), 7.20 (d, J = 7.6 Hz, 1H), 5.18 (t, J = 6.3 Hz, 1H), 3.08 (q, J = 6.4 Hz, 2H), 2.89 (s, 6H), 2.51 (dt, J = 8.7, 6.3 Hz, 2H), 1.21 (t, J = 8.7 Hz, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 152.1, 150.1, 134.6, 130.7, 129.9, 129.6, 129.5, 128.6, 123.2, 118.5, 115.3, 45.9, 45.4, 24.8. ESI (+) MS m/z 311.2 [M + H]⁺.

Instrumentation and data analysis

The fluorescence quantifications were performed on a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, California, USA). Data were acquired and processed using SoftMax Pro 5 (Molecular Devices, Sunnyvale, California, USA) and Microsoft Excel 2013 software.

Experimental procedure

In a 2 mL microcentrifuge tube, 50 μ L of 2.5 mM DCYA (1 equiv.) was mixed with 50 μ L of a 5 mM solution of test electrophile (2.0 equiv.), both prepared in acetonitrile (ACN). Twenty microliters of aqueous pH 10 buffer was added to the solution and incubated for 20 min at room temperature. A set of three controls was prepared as summarized in Figure 2C (further discussion on the controls is provided in the Results section). Blank samples (Bl) were prepared by adding 20 μ L of ACN in place of the buffer. Fifty microliters of DCYA mixed with 70 μ L of ACN was used for the Positive Control (PC). Negative Controls (NC) were prepared by adding 50 μ L ACN to 50 μ L DCYA then incubating with 20 μ L buffer. Silica-supported maleimide (20 mg) was then added to remove unreacted DCYA, and the suspension was vortexed and incubated for 1 h with vigorous agitation. The samples were then centrifuged at 14,000 rpm (20 sec), diluted to a final volume of 1 mL with ACN, vortexed, and centrifuged for 10 min at 14,000 rpm. Each sample was then transferred to a solvent-resistant microplate (100 μ L sample + 100 μ L ACN). The samples were evaluated using end-point readings and full spectrum profiles for fluorescence and absorbance. Fluorescence end-point readings were acquired at 520 nm (excitation 350 nm, cutoff at 420

nm), temperature 23 °C, 50 readings (high sensitivity). Full emission spectra were recorded over a range of emission wavelengths from 420 to 700 nm (excitation 350 nm, no cutoff). Absorbance profiles were recorded on a range 300-750 nm. The stock solutions of DCYA were tested for fluorescence response immediately before use. Stock solutions of DCYA which showed loss of fluorescence >5% compared to freshly prepared solutions were discarded.

Statistical analysis

Cooper statistics were performed on binary classification of sensitizers (excluding pre- and pro-haptens) by summing the number of compounds classified as “true positive” (TP), “false positive” (FP), “true negative” (TN) and “false negative” (FN):

$$\text{Sensitivity (\%)} = 100 * \frac{TP}{TP+FN}$$

$$\text{Specificity (\%)} = 100 * \frac{TN}{TN+FP}$$

$$\text{Positive predictive value (\%)} = 100 * \frac{TP}{TP+FP}$$

$$\text{Negative predictive value (\%)} = 100 * \frac{TN}{TN+FN}$$

$$\text{Accuracy (\%)} = 100 * \frac{TP+TN}{TP+FP+TN+FN}$$

Thin Layer Chromatography

A two microliters aliquot of each sample (before the incubation with silica-supported maleimide) was loaded on a silica gel plate and eluted with 0.8% MeOH in CHCl₃ or other appropriate solvent systems as needed (Supporting information) to achieve adequate resolution. The TLC plates were examined at λ_{366} for the presence of the yellow fluorescent spots corresponding to DCYA (R_f 0.33 in the mentioned solvent conditions), its dimer (R_f 0.18) or possible reaction products. (confirmed by HPLC/MS, data not shown).

Results

A schematic workflow representation of the proposed method is given in Figure 1. Several experimental parameters were assessed in order to determine the influence of each parameter on the reaction rate and fluorescence response.

Excitation, Emission and Calibration of DCYA

The fluorescence responses of DCYA and DCYA disulfide in ACN were investigated at different concentrations using 200 μL /well in a 96 microplate reader at a constant temperature (23 $^{\circ}\text{C}$). Pure acetonitrile was used as a control. The optimum excitation and emission wavelength combination was determined by performing excitation and emission scans at 2 nm intervals. Regardless of test concentration, both DCYA and its disulfide were found to have excitation and emission maxima at 350 and 520 nm, respectively. At these wavelengths, no solvent interference was observed. However, 1.5-3.0% loss of fluorescence response was observed for micromolar solutions of DCYA after 3 h at room temperature. A linear response for DCYA was observed between 5-80 μM (10-160 nmol/well) concentrations with a $R^2 = 0.995$. Fluorescence emission and excitation spectra, absorbance profile and calibration curves for DCYA are shown in Figure S1 (Supplementary content).

Solvent effect

Several solvent systems were tested, including the polar aprotic solvents ACN and dimethyl sulfoxide (DMSO) along with chlorinated solvents. Even though chloroform and DMSO are suitable solvents for nucleophilic additions (Chittiboyina et al., 2015), they were here excluded because of issues with evaporation, and interference with the detection mode. Acetonitrile was thus chosen as the preferred solvent because of its low oxidative effect on DCYA, relatively high boiling point and adequate solvent strength. The majority of the electrophilic compounds are highly hydrophobic in nature (Bos and Meinardi, 2000) and the use of organic solvents minimize the risk of drowning-out effects. The use of a two-solvent system has been previously suggested (Gerberick et al., 2004b; Natsch et al., 2007; Natsch

and Gfeller, 2008) but care should be taken in the comparison of results obtained in different solutions, because the solvent may have a strong influence on the extent of reaction. In some cases, especially with DMSO, the possibility of a pro-oxidation effect should be taken into account, as it may dramatically affect the quantification of the reacted nucleophile (Böhme *et al.*, 2009).

Catalysis and the role of controls

Because the reactivity of DCYA is dependent on the activation of the thiol by base catalysis, the reaction rate in the presence of organic or inorganic bases was examined. The known sensitizer parthenolide (P), the small lipophilic natural terpene massoia lactone (ML) and the non-sensitizer coumarin (COUM) were chosen as model electrophiles to determine the degree of reaction. The organic base 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) (pKa 13.5) and aqueous pH 10 buffer gave comparable results in preliminary studies (Chittiboyina *et al.*, 2015), providing adequate conditions for activation of the thiol and further addition with test compounds. Parameters, such as concentration of the electrophile, base, dilutions, and the incubation time, were further optimized for the fluorescence assay. To gain some insights about the influence of each parameter, thin-layer chromatography experiments were conducted as a pictographic aid. Concentrations of DBN solutions (prepared in acetonitrile) from 0.1 to 0.2 equiv. were insufficient to catalyze a complete conversion of parthenolide under the chosen conditions (Figure S2), while higher conversion was observed with 0.4 equiv. DBN or with pH 10 buffer. No substantial adduct was observed when a pH 7 buffer was used. The same buffer conditions (pH 10) were further explored in the fluorescence assays in microplates after optimization of the thiol scavenging conditions. In order to calculate the net fluorescence derived from DCYA-electrophile adducts, positive and negative controls were included (Material and Methods). No DCYA anion was expected in the absence of base (PC). The neutral thiol should not react with the maleimide scavenger and the maximum recovery fluorescence in the supernatant was expected. In the negative control, DCYA and its conjugate base, DCYA⁻ would be in equilibrium. The resulting thiolate would bind to the silica-supported maleimide, and no fluorescent species should remain in the solution. The residual fluorescence read for the NC was used to calculate the

buffer effect in the absence of any reaction. This non-specific response was correlated to the dimerization of DCYA under basic conditions. The PC and NC reactions were treated with an excess of maleimide, and diluted so that the fluorescence reading would fit within the linear range of the calibration curve. Ideally, low NC/PC ratios are desired. As shown in Table S1, DBN concentrations up to 0.4 equiv. were insufficient to promote an efficient scavenging, whereas the best scavenging effect was obtained with 16% (v/v) pH 10 buffer in acetonitrile.

Reaction time and electrophile stoichiometry

The reaction time is a critical factor, since thiols are known to auto-oxidize to disulfides under basic conditions. In order to minimize the competition between auto-dimerization [(DCYA)₂] and nucleophilic addition, the reaction time and the electrophile:nucleophile stoichiometry were optimized. Massoia lactone at different concentrations was used to monitor the progress of the reaction by TLC every 5 minutes. In the presence of the electrophile, the formation of DCYA-ML adduct was dominant with only a negligible amount of (DCYA)₂ present (Figure 2). In the absence of electrophile (NC), the formation of (DCYA)₂ was minimal for reaction times below 25 minutes, while increased amount of disulfide was observed with longer incubation times. In the presence of strong electrophiles (P), the reaction product was dominant at the lower concentration tested (1 equiv.), while only a partial reaction was achieved in the presence of 1 equiv. ML (Figure S3). In the case of a non-sensitizer (COUM, Figure S4), no reaction was observed at the concentrations tested. In order to adequately discriminate between compounds with variable electrophilic strength, the method was optimized using 2 equivalents of electrophile. Higher concentrations may be useful to better discriminate weak and extremely weak electrophiles from non-sensitizers, but may also lead to overestimation of reactivity for moderate electrophiles. Lower concentrations may negatively affect the sensitivity of the method, especially in the presence of borderline reactive compounds.

Thiol scavenger

Maleimide supported on silica is a good thiol scavenger possessing high selectivity for thiols over other types of nucleophiles. Silica-supported maleimide was found to readily and selectively bind to DCYA⁺ but not to non-activated DCYA under the tested conditions. The effectiveness of the maleimide scavenger was also pH dependent, and optimum conditions were determined using 20 mg of maleimide (0.17 mg /nmol DCYA) in the presence of 16% v/v aqueous pH 10 buffer in acetonitrile (Table S1). One hour incubation was found to be sufficient to obtain a maximum scavenging effect, while negligible improvements were observed for 2 or 3 h incubations.

Controls

In order to minimize the risk of over or under estimation of the reaction adducts, two controls were included in each sample set to calculate the maximum (PC) and minimum (NC) fluorescence response (Figure 2C). The fluorescence response of the controls was measured using end-point reading at a fixed concentration (62.5 μ M DCYA in ACN), with or without incubation with silica-supported maleimide. Recovery greater than 95% was obtained for the PC under the tested conditions. The average inter-day variation of the NC fluorescence response was between 23-27% of the maximum reading (PC). A “blank” control (Bl) was used to determine the fluorescence interference (background noise) of each electrophile on the reaction. The blank reading was obtained by combining the electrophile with DCYA in the absence of catalyst (Figure 2A). Under these conditions, no reaction should occur and the fluorescence response (RFU) should be close to the positive control (if no electrophile interference is observed). The reaction (R) readings were performed by mixing 2.0 equiv. of electrophile and DCYA in the presence of pH 10 buffer. Reactive electrophiles generated higher fluorescence responses as determined by the reaction equilibrium (Figure 2B). All the controls and the reactions were performed in triplicate and each plate was read at least three times, thus obtaining a total of 9 readings per sample.

Data analysis

A minimum of 9 readings were averaged per sample, and an example of the raw data obtained is reported in Table S2 (Supplementary content). Inter-day and batch-to-batch variations were assessed on standard solutions at 62.5 μ M of DCYA (in RFU) over a two-year period and found to vary on average $\leq 5\%$ in either case (Tables S3 and S4). The degree of reaction was calculated based on the comparison between the reaction response and the controls according to the formula

$$RI = 100 \times \left(1 - \frac{Bl - R}{PC - NC} - \frac{PC - Bl}{PC} \right)$$

where the *reactivity index (RI)* is proportional to the reactivity of the candidate sensitizer. Caution should be taken when using the *RI* value to determine the extent of the reaction: although a direct relationship between the reactivity and the *RI* exists, an *RI* of 100 does not necessarily mean 100% reaction. Depending on inter-day and batch-to-batch variations, *RI* results varied on average less than 5 points, thus a standard error of $RI \pm 5$ has been considered acceptable.

Method validation

In order to compare the performance of the fluorescence assay with existing methods for skin sensitization assessment, a set of known sensitizers was investigated. The list includes 36 natural and synthetic compounds from the “Silver-list” (Natsch *et al.*, 2013) covering a range of diverse mechanistic domains and sensitization potential. Compounds lacking requisite mechanistic domains, such as hydroquinone or cinnamyl alcohol, (Niklasson *et al.*, 2014) have also been included in the list, but they have been excluded from the statistical analysis because the impossibility of identifying pre- or pro-sensitizers using *in chemico* approaches is a known limitation if not combined with chemical or enzymatic activation (as in the Peptide Peroxidase Reactivity Assay) (Gerberick *et al.*, 2009). The results of the DCYA assay are presented in Table 1 along with Cys-DPRA results abstracted from the literature. For comparison with the Cys-DPRA (which are given as percentage of remaining peptide), results are reported as 100-*RI* to estimate the remaining unreacted DCYA. A color coded classification of potency is presented merely for illustrative purposes. It is worth to remember at this point that only LLNA has been

validated for potency classification in the absence of human data. *In vitro* methods including DPRA and KeratinoSensTM can only provide approximate information on the potency but such information is *not* sufficient for classification purposes. Chemical methods are essential to estimate the potential to induce later haptenation events by covalently binding to the biological target, but cannot serve as a standalone alternative to animal tests. Better predictions can be achieved when chemical reactivity results are integrated with cell-based approaches (Urbisch *et al.*, 2015). Indicative values for DPRA and KeratinoSensTM used herein for classification purposes are reported in Table 2.

As expected, the putative pre- or pro-haptens hydroquinone, resorcinol and hydroxytyrosol (Kern *et al.*, 2010), were found to be not reactive in both Cys-DPRA and DCYA assays. The cyclic anhydride, 1,2-cyclohexanedicarboxylic anhydride, is a known respiratory allergen with higher selectivity for lysine than cysteine (Lalko *et al.*, 2013). Occurrence of contact urticaria to cyclic anhydrides is rare compared to the widespread cases of respiratory allergy, although cases of contact sensitization have been reported as occupational hazards (Helaskoski *et al.*, 2009). In the case of *p*-benzoquinone (BQ), a complete reaction was confirmed by TLC but the final quantification was affected by the instability of the compound, with the formation of precipitates during the reaction time. In this case, BQ was thus correctly identified as a sensitizer (Roberts and Aptula, 2009) but the degree of reaction has been underestimated. Lilial was found non-reactive in the DCYA assay, and these finding correlated with the KeratinoSensTM data. Bromobutane and benzaldehyde were classified as non-reactive by the DCYA method, while a minimal reaction was found with Cys-DPRA. Bromobutane is considered a false positive in Cys-DPRA. A minor DCYA adduct with bromobutane was observed by HPLC/MS after 24 h of incubation; this case exemplifies the risk of overestimation of the nucleophile depletion during long incubation periods. In the case of benzaldehyde, no DCYA adducts were identified, and the possibility of over-estimation due to the pro-oxidant effect of benzaldehyde should be considered. The aromatic compounds 1-chloro-2,4-dinitrobenzene (CDNB) and diphenyl cyclopropanone (DPCP) were classified as false negative in DCYA. However, a complete reaction of CDNB with DCYA was confirmed by TLC, where no unreacted DCYA and only a very low amount of disulfide was observed along with a non-fluorescent DCYA-

CDNB adduct. The DCYA-CDNB adduct has been isolated and characterized by NMR and mass spectrometry, thus confirming CDNB as a strong reactive electrophile (Figure S5).

The performance of the DCYA and Cys-DPRA results was compared by Cooper statistical analysis (Tables 3 and 4). Chemicals with a $RI \geq 5$ (Table 1) were classified as positive skin sensitizers in the DCYA assay. Within the limited set of sensitizers tested, the DCYA gave comparable results with the Cys-DPRA in terms of accuracy (81.8% and 92.6%, respectively). A tendency to overestimation of the reactivity of moderate electrophiles in the DPRA was found, especially in the presence of potential pro-oxidizers. This observation was confirmed by the higher number of false positives in DPRA than DCYA (Table 3). On the other hand, mildly reacting electrophiles with very weak or borderline reactivity (e.g. perillaldehyde, moderate sensitizer in validated methods) were underestimated using the DCYA method. This drawback could be addressed by measuring the DCYA depletion at increasing concentrations of the test chemical. As this work focused mainly on the development of a rapid and inexpensive method to maximize the number of simultaneously tested compounds, such an approach was beyond the scope of this study. The majority of strong electrophiles displayed a $100-RI < 20$, whereas moderate electrophiles measured in the range 20 to 80 thus such values were here used as approximate threshold for potency classification. Further validation on a larger set of compounds is needed for more accurate statistical analysis and is currently ongoing.

Along with the known set of sensitizers, 6 natural compounds not included in the “Silver-list” were investigated and their potential reactivity was evaluated (Table 5). The three lactones (costunolide, parthenolide and alantolactone) are among the most common contact allergens isolated from plants (Mitchell and Dupuis, 1971; Hausen, 1981; Hausen, 1991), and they are constituents of the sesquiterpene lactone mix (Jacob *et al.*, 2012) and the Compositae mix used in clinical patch tests (Paulsen *et al.*, 2001). Costunolide, alantolactone and parthenolide were found to be equally strongly reactive. The small natural aldehyde *t*-2-pentenal was classified according to DCYA results as moderate reactive, with an $RI-100$ close to the homologous *t*-2-hexenal, which was also found as moderate sensitizer in LLNA (Nukada *et*

374 *al.*, 2013). Finally, the two volatile Michael acceptors ML and nootkatone were determined to have RI-
375 100 values of 12.8 and 96.5, respectively.

376 377 Discussion

378 The aim of the present work was the development of a fast and sensitive method to determine the
379 electrophilic reactivity of potential skin sensitizers. To achieve this goal, a fluorescent dansylated
380 cysteamine (DCYA) was designed as a model nucleophile. Dansyl derivatives are widely used in
381 fluorescence spectroscopy, especially for protein and amino acids labeling, as well as Zn^{2+} and Fe^{3+}
382 detection (Yang *et al.*, 2013). One of the major advantages of dansyl fluorophores is their relative
383 stability, the high quantum yield and large Stokes shift, with a maximum emission usually in the 500 nm
384 range. These features allowed the development of a highly sensitive fluorescence assay optimized for
385 HTS. Cysteamine was chosen as the model nucleophile. Cysteamine is a versatile lipophilic cysteine
386 analogue compatible with organic solvents (Avonto *et al.*, 2011). The short aliphatic chain provided a
387 suitable linking distance between the reactive thiol and the dansyl group without interfering with the
388 electronic push/pull mechanism of the fluorescent tag. The synthesized DCYA was found to be stable for
389 at least 4 months when stored at -4 °C avoiding light and oxygen exposure.

390 Compared to similar *in chemico* assays, the HTS-DCYA can be considered innovative at three
391 levels: the detection method, the use of a model lipophilic nucleophile and the direct quantification of the
392 reaction adducts. Only a minimal amount of reagents are required, which is a desirable advantage in
393 routine screenings of large compilation of chemicals. The fluorescence detection at 520 nm ensured a
394 good signal/noise ratio with minimal interferences. The developed reaction procedure allows
395 minimization of the reaction time and maximization of the throughput compared to the 24 h reaction time
396 needed for existing assays. In such a short time period, generation of by-products is also quite limited
397 even in the presence of pro-oxidants. Since hydrophobicity is a pre-requisite of sensitizers ($\log P_{o/w} > 1$)
398 (Smith Pease *et al.*, 2003), the use of an organic solvent may be more desirable to avoid precipitation
399 issues that could influence the final quantification. Thirty-two samples (15 samples, 15 blanks and 2

controls) were analyzed in triplicate in a 96 well plate and the conditions are potentially adaptable for higher throughput, thus further reducing the amount of reagents, waste, time and costs of screenings of large chemical libraries. The presence of three sets of controls reduces the risk of overestimation of the reaction yield and thus of false positives. A comparison of DCYA reactivity assays with Cys-DPRA showed a degree of correlation between the two methods (Table 1). A trend toward higher depletion of nucleophile with increasing sensitizer potency was found in both chemical assays, with the obvious exception of pre- or pro-haptens, which lack reactive mechanistic domains. The discrimination across chemicals with a wide reactivity range can provide useful insights on the potency of the candidate sensitizer (Chittiboyina et al., 2015). Nonetheless, it should be emphasized that there is still an ongoing debate whether (and to what extent) reactivity alone can be used to predict potency, especially when comparing results across different mechanistic domains (Natsch et al., 2014).

In conclusion, the newly developed DCYA assay may represent an additional tool for *in chemico* characterization of potential skin sensitizers. The sample throughput and minimal amounts required makes the DCYA method a fast and economical approach that may be useful in pre-screenings of large chemical libraries before performing more costly and time-consuming *in vivo* sensitization tests.

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Figures and Tables

Figure 1. Schematic workflow of the HTS-DCYA assay.

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Reagent	Positive control	Negative control	Blank	Reaction
DCYA	YES	YES	YES	YES
Electrophile	NO	NO	YES	YES
Buffer	NO	YES	NO	YES
Scavenger	YES	YES	YES	YES
Evaluation	Max. response	Min. response	Electrophile interference	Reaction

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Figure 2. Schematic representation of reaction between DCYA and Massoia lactone. In the absence of a base (blank, A) the reactivity of DCYA is negligible and no adducts are expected in the short time (20 min). Addition of a base (reaction, B) generates a reactive thio-anion which quickly reacts with potential sensitizers. The extent of such reaction is determined by the reactivity of the electrophile. Figure 2C summarizes the conditions used for controls, blanks and reactions (Material and Methods).

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TABLE 1.

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Reactivity and classification of known sensitizers in HTS-DCYA assay and data comparison with

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reported methods.

Chemical	CAS NO.	DCYA	Cys-DPRA ¹	KeratinoSens TM	LLNA ¹
		100-RI	% Pept. Remain.	EC ₃ (μM)	EC ₃
Diphenyl cyclopropenone	886-38-4	96.3	1.2	1.84	0.003
<i>p</i> -Benzoquinone	106-51-4	34.5	1.0	32.77	0.01
1-Chloro-2,4-dinitrobenzene	97-00-7	131.7	0.0	3.89	0.05
<i>p</i> -Hydroquinone ^a	123-31-9	127.1	16.7	51.29	0.11
Propionolactone	57-57-8	64.9	n.a.	n.a.	0.2 ²
3-Hydroxytyrosol ^a	10597-60-1	97.2	n.a.	n.a.	0.6 ³
1,2-cyclohexanedicarboxylic anhydride ^b	85-42-7	104.6	84.3	>2000	0.84
2-Methyl-4-isothiazolin-3-one	2682-20-4	16.4	2.1	29.56	1.9
Cinnamaldehyde	14371-10-9	85.1	29.4	63.94	3.0
2,4-Heptadienal	5910-85-0	54.1	2.7	21.91	4
4-Hex-3-en-one	2497-21-4	2.8	n.a.	n.a.	4.2
Squaric acid	2892-51-5	91.1	53.1	>2000	4.3
<i>l</i> -2-Hexenal	6728-26-3	29.9	2.1	374.57	5.5
Resorcinol ^a	108-46-3	103.5	98.4	>2000	5.5
Diethyl maleate	141-05-9	8.84	0.0	82.85	5.8
Safranal	116-26-7	82.9	8.2	n.a.	7.5
Perillaldehyde	2111-75-3	95.8	68.1	61.85	8.1
Citral	5392-40-5	88.8	14.3	67.36	9.2
Farnesal	502-67-0	94.8	83.6	>2000	12
L-Carvone	6485-40-1	88.4	n.a.	n.a.	13 ⁴
Oxalic acid	144-62-7	89.4	99.1	>2000	15
Benzyl Benzoate ^a	120-51-4	110.6	99.8	142.47	17
Lilial ^a	80-54-6	98.9	86.0	>2000	19
Cinnamyl alcohol ^a	104-54-1	105.0	100	>2000	21
<i>cis</i> -6-Nonenal	2277-19-2	107.3	92.0	687.67	23
5-Methyl-2,3-hexanedione	13706-86-0	93.7	74.2	332.21	26
Ethyl acrylate ^c	140-88-5	-0.7	3.6	231.19	28
Aniline	62-53-3	107.0	100	>2000	89
1-Bromobutane	109-65-9	100.0	86.2	>2000	NC
Vanillin	121-33-5	106.4	96.8	>2000	NC
Tartaric acid	87-69-4	100.7	96.2	>2000	NC
Chlorobenzene	108-90-7	97.8	99.6	>2000	NC

Lactic acid	50-21-5	100.1	100.0	>2000	NC
Salicylic acid	69-72-7	106.9	96.5	>2000	NC
Coumarin	91-64-5	97.2	99.0	479.96	NC
Benzaldehyde	100-52-7	98.1	92.8	>2000	NC

Non reactive; Weak; Moderate Strong/extreme (color coded for illustrative purposes only)

¹ Natsch, 2013 ² Gerberick 2004a ³ Kern 2010 ⁴ Natsch, 2011

^a Putative pre/pro-hapten; ^b Selective toward Lys-DPRA; ^c False negative in LLNA; NC = Non Classified

TABLE 2

Classification	DCYA (100-RI)	Cys-DPRA ¹ (% Pept. Remain.)	KeratinoSens ^{TM 7*} EC3 (µM)	LLNA ¹ EC3 (%)
Strong/Extreme	<20	<57.73	39.8	<0.1 (extreme) 0.1-1 (strong)
Moderate	20-80	57.73-77.38	133.3	1-10
Weak	80-95	77.38-93.62	2000	≥10
Non sensitizer	>95	>93.6	>2000	NC

*Median value ¹Natsch, 2013

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TABLE 3

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Binary classification of potential sensitizers in DCYA and DPRA.

Chemical	CAS NO.	DCYA				Cys-DPRA			
		TP	TN	FP	FN	TP	TN	FP	FN
Diphenyl cyclopropenone	886-38-4	0	0	0	1	1	0	0	0
<i>p</i> -Benzoquinone	106-51-4	1	0	0	0	1	0	0	0
1-Chloro-2,4-dinitrobenzene	97-00-7	0	0	0	1	1	0	0	0
Propionolactone	57-57-8	1	0	0	0	n.a.	0	0	0
2-Methyl-4-isothiazolin-3-one	2682-20-4	1	0	0	0	1	0	0	0
Cinnamaldehyde	14371-10-9	1	0	0	0	1	0	0	0
2,4-Heptadienal	5910-85-0	1	0	0	0	1	0	0	0
4-Hex-3-en-one	2497-21-4	1	0	0	0	n.a.	0	0	0
Squaric acid	2892-51-5	1	0	0	0	1	0	0	0
<i>t</i> -2-Hexenal	6728-26-3	1	0	0	0	1	0	0	0
Diethyl maleate	141-05-9	1	0	0	0	1	0	0	0
Safranal	116-26-7	1	0	0	0	1	0	0	0
perillaldehyde	2111-75-3	0	0	0	1	1	0	0	0
Citral	5392-40-5	1	0	0	0	1	0	0	0
Farnesal	502-67-0	1	0	0	0	1	0	0	0
L-Carvone	6485-40-1	1	0	0	0	n.a.	0	0	0
Oxalic acid	144-62-7	0	0	1	0	0	1	0	0
Benzyl Benzoate	120-51-4	0	1	0	0	0	1	0	0
Lilial	80-54-6	0	0	0	1	1	0	0	0
cis-6-nonenal	2277-19-2	0	0	0	1	1	0	0	0
5-Methyl-2,3-hexanedione	13706-86-0	1	0	0	0	1	0	0	0
Ethyl acrylate	140-88-5	1	0	0	0	1	0	0	0
1-Bromobutane	109-65-9	0	1	0	0	0	0	1	0
Vanillin	121-33-5	0	1	0	0	0	1	0	0
Tartaric acid	87-69-4	0	1	0	0	0	1	0	0
Chlorobenzene	108-90-7	0	1	0	0	0	1	0	0
Lactic acid	50-21-5	0	1	0	0	0	1	0	0
Salicylic acid	69-72-7	0	1	0	0	0	1	0	0
Coumarin	91-64-5	0	1	0	0	0	1	0	0
Benzaldehyde	100-52-7	0	1	0	0	0	0	1	0
Costunolide	553-21-9	1	0	0	0	n.a.	n.a.	n.a.	n.a.
Alantolactone	546-43-0	1	0	0	0	n.a.	n.a.	n.a.	n.a.
Parthenolide	20554-84-1	1	0	0	0	n.a.	n.a.	n.a.	n.a.
TOTAL		18	9	1	5	17	8	2	0

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TP = True Positive; TN = True Negative; FP = False positive; FN = False Negative

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Yes= 1, No = 0, n.a. = not available

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TABLE 4.

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Cooper statistics for DCYA and Cys-DPRA assays.

	DCYA	Cys-DPRA
Positive predicted values	94.7	89.5
Negative predicted values	64.3	100.0
Accuracy	81.8	92.6
Sensitivity	78.3	100.0
Specificity	90.0	80.0
N	33	27

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TABLE 5.

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DCYA results for six chemicals not included in the Silver list.

Chemical	CAS NO.	100-RI	Reference and comment
Costunolide	553-21-9	-8.5	Ducombs, 1990 ^a
Alantolactone	546-43-0	-6.7	Ducombs, 1990 ^a
Parthenolide	20554-84-1	0.5	Ducombs, 1990 ^a
Massoia lactone	51154-96-2	12.8	-
<i>trans</i> -2-Pentenal	1576-87-0	18.2	-
Nootkatone	4674-50-4	96.5	-

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^a Positive in human patch tests

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